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Title：The changes in metastatic potential of radioresistant cancer cell line generated by frequent X-ray

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Abstract

In general, cancer cells which survived from X-ray irradiation have acquired radioresistance. These survived cells are cause of recurrence and/or metastasis. The purpose of this study is to evaluate metastatic potential of the cancer cells survived from frequent X-ray irradiation. The adhesiveness in A549R was remarkably increased than in A549. The motility of A549R was significantly suppressed than A549. There was no significant difference in invasiveness. Integrin β1 expression of A549R was more increased than A549. In comparison with non-irradiated each cell line, adhesiveness in both cell lines were most increased in 2 hours or 24 hours. The motility of A549R was most increased in 2 hours compared with non-irradiated cell lines whereas motility of A549 was most increased in 24 hours. As compared with non-irradiated cell lines, invasiveness in both cell lines was significantly suppressed. However, there was no prominent difference in temporal change in both cell lines. The adhesion ability of A549R was more increased than A549 consistent with increased integrin β1 expression. Our study demonstrates that radio-resistant cancer cell line increase the adhesion capability consistent with enhance the expression integrin β1 and suppress the cell migration. The change in radio-resistant cancer cell line takes place at the time adhesive capacity and cell migration was most increased compared with parental cancer cell line after irradiation. When radio-resistant mechanism becomes clear, a new treatment policy is established. This leads shortening of the treatment period, and, as a result, medical expenses are reduced. In future, it is necessary to investigate the effective radiotherapy that consider the effect for health economics.

Keyword：X-ray, radioresistant cell, adhesion, motility, invasion, reduce the medical expense

The changes in metastatic potential of radioresistant cancer cell line generated by frequent X-ray

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Abstract

In general, cancer cells which survived from X-ray irradiation have acquired radioresistance. These survived cells are cause of recurrence and/or metastasis. The purpose of this study is to evaluate metastatic potential of the cancer cells survived from frequent X-ray irradiation. The adhesiveness in A549R was remarkably increased than in A549. The motility of A549R was significantly suppressed than A549. There was no significant difference in invasiveness. Integrin β1 expression of A549R was more increased than A549. In comparison with non-irradiated each cell line, adhesiveness in both cell lines were most increased in 2 hours or 24 hours. The motility of A549R was most increased in 2 hours compared with non-irradiated cell lines whereas motility of A549 was most increased in 24 hours. As compared with non-irradiated cell lines, invasiveness in both cell lines was significantly suppressed. However, there was no prominent difference in temporal change in both cell lines. The adhesion ability of A549R was more increased than A549 consistent with increased integrin β1 expression. Our study demonstrates that radio-resistant cancer cell line increase the adhesion capability consistent with enhance the expression integrin β1 and suppress the cell migration. The change in radio-resistant cancer cell line takes place at the time adhesive capacity and cell migration was most increased compared with parental cancer cell line after irradiation. When radio-resistant mechanism becomes clear, a new treatment policy is established. This leads shortening of the treatment period, and, as a result, medical expenses are reduced. In future, it is necessary to investigate the effective radiotherapy that consider the effect for health economics.

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Introduction

Radiotherapy is an effective approach for treatment of many types of cancers. Recent developments in radiotherapy technology, such as intensity-modulated radiation therapy (IMRT) and three-dimensional (3D) radiotherapy, allow precise dose escalation to the tumor. It has improved local control rates [1, 2].

Non-small cell lung cancer (NSCLC) is one of the most common causes of mortality in Japan, accounting for approximately 80 % of all lung cancer patients. Radiotherapy is the most commonly used treatment option for medically inoperable NSCLC. Many reviews have summarized report on early stage NSCLC after fractionated radiotherapy [3-5]. Sibley reported that approximately 30 % of patients died from distant metastasis [3]. This poor prognosis is due to survive from radiotherapy. In addition, some studies have reported that cancer cells after irradiation acquired radioresistance [6, 7]. There is a possibility that the cancer cell survived from X-ray irradiation and acquired radioresistance promotes metastasis. Thus, poor prognosis in NSCLC after treatment will be associated with that cell. In the clinic, most of facilities adopt the conventional planning, which is approximately 2 Gy × 30 fractions (total 60 Gy). Clarification of features of radioresistant cancer cell due to survive from radiation therapy is important to improve survival rate of cancer patients. We has established the radio-resistant cell line named A549R which was irradiated with 2 Gy/fraction, total 60 Gy with 4 MV X-ray over 6 months, and radioresistant cell line was cloned.

In this study, we investigate metastatic potential of radio-resistant cancer cell line and this cell line is compared with parental cell line.

Materials and Methods

1. Cell culture

A549 human lung adenocarcinoma cell and A549R radio-resistant cancer cell line were maintained in DMEM medium (Wako, Osaka, Japan) with 10 % fetal bovine serum (FBS) (Biowest, France, Nuaillé) and penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan) at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. The establish methods of radiation resistant cell line were described previously. Briefly, A549 was irradiated with 2 Gy/ fraction, 30 fractions with 4 MV X-ray over 6 months. Therefore total dose 60 Gy was delivered. After the treatment, the radio -resistant clone A549R is isolated and cloned from the irradiated subclone. We have confirmed that the radiation sensitivity is significantly lower than that of A549.

2. Irradiation

A549 and A549R were cultured with 10 % fetal bovine serum and 0.025 % penicillin / streptomycin containing DMEM in 5 % CO_2 and 95% air. The day before radiation treatment, each cell was prepared to cell culture dishes. X ray irradiation was performed with medical linear accelerator (LINAC) at Osaka University Graduate School of Medicine with delivering dose rate of 1.0 Gy/min. For X-ray irradiation, FBS free DMEM was added to form build-up before irradiation.

3. Cell Adhesion Assay

A 96-well plastic plates were coated with 10 μg/ml of collagen IV (BD, 354233, Mississauga, Canada), laminin (BD, 354232, San Jose, CA)

fibronectin (BD, 354008, San Jose, CA) or vitronectin (BD, 354238, San Jose, CA) in phosphate-buffered saline (PBS) (Gibco, New York, USA) for 2 h at 37°C and then treated with 3 % bovine serum albumin (BSA) for 1 h at 37°C, or were coated with only BSA for negative control. 2.5×10^4 cells in FBS free DMEM containing 0.1 % BSA were added and incubated for 2 h at 37°C. After removal of the medium, a 0.04% crystal violet solution was added and incubated for 10 min at room temperature. The wells were washed three times with PBS and 20 μL of dimethyl sulfoxide were added for permeabilization. Distilled water was then added to 100 μL, and the number of adherent cells was assessed with a microplate reader (measurement wavelength 550 nm and reference wavelength 630 nm).

4. Boydenchamber Assay

The cells were collected by using trypsin-EDTA in PBS and suspended with serum-free medium containing 0.1 % BSA after the cells had been washed with the same medium. The migratory activities of A549 and A549R cells through 8-μm pores were assessed. A filter was placed on a 24-well plate and coated with fibronectin for 30 min at room temperature. DMEM supplemented with 10 %FBS was used as a chemoattractant into the lower wells. Thirty thousand of untreated or treated cells were plated onto the filter membrane and the allowed cells to migrate through the membrane at 37°C in an atmosphere of 5 % CO_2. After 3 hours, not migrated cells were scraped off with a cotton swab, and the filter membrane was removed with a blade. The cells that had migrated to the bottom side of the membrane were fixed with 10 % formalin and then stained with hematoxylin. Cell migration was quantitated by counting the number of stained with hematoxylin in three random fields at 200 × magnification with a microscope.

5. Matrigel invasion Assay

Invasion of cancer cells was measured by the invasion of cells through transwell inserts with 8-μm pores that were coated with Matrigel (Becton Dickinson, California, USA). Irradiated cells were trypsinized, washed two times with DMEM supplemented with 0.1 %BSA, and 200 μL of cell suspension (5×10^5 cells/ml) were added to the upper well. DMEM supplement with 700 μL of 10 % FBS as a chemoattractant was added to the lower well. The number of cells that had invaded to the lower surface of the Matrigel-coated membrane was counted in three random fields under a microscope.

6. Integrin expression analysis using flowcytometery

The cells were collected with Trypsin-EDTA. The cells were dissolved in 0.1 % FBS and 0.03 % NaN3. Three hundred thousand cells were added in the 96-well plate. The following processes were done at 4℃. 0.1% FBS and 0.03 % NaN_3 were used as the washing solution. Anti integrin β1 antibody (MAR4) (BD, 555442, San Jose, CA) was diluted 1:100 with washing solution, and used as primary antibody. As the control, washing solution of 100 μL was added. After 30 minutes, it was washed three times with the solution. Secondary anti-mouse IgG (DAKO, F0261, Copenhagen, Denmark) of the undiluted solution and 100 μL of washing solution was added. Mouse immune serum was used as the secondary antibody of β1.

After 30 minutes, it was washed three times. Flow Cytometry analysis was performed using FACS Calibur (BECTON DICKNSON Co.) and integrin expression on the cell surface was measured.

7. Statistics

The results were expressed as mean values with standard deviations. The statistical significance was tested by means of Student's t-test. A p-value of less than 0.05 was considered to be statistically significant.

Results

1. Difference in metastatic potential between A549 and A549R

Cell adhesion, migration, and invasion capabilities and integrinβ1 expression in A549 and A549R are shown in Figure 1. Adhesion ability to extracellular matrix in A549R was significantly increased compared with A549 (Fig. 1A). Cell migration in A549R was suppressed than A549 (Fig. 1B). There was not difference in cell invasion capability between both cells (Fig. 1C). Integrin β1 expression in A549R was promoted, compared with expression of integrin β1 in A549 (Fig. 1D).

2. Effects of irradiation on cell adhesion capabilities

Cell adhesion ability of A549 and A549R to each extracellular matrix in 2, 6, 12, 24 hours after X-ray irradiated is shown in Figure 2. Adhesion capability to collagen IV in A549R was most increased by about in 2 hours 160 % after X-ray irradiated whereas in A549 was most promoted by about 210 % in 24 hours after irradiation compared with non-irradiated each cell line (Fig. 2A). With fibronectin, the same tendency as collagen IV was observed (Fig. 2B). In comparison with non-irradiated each cell line, adhesion ability on laminin in A549 and A549R cells was most increased by about 220 % and 170 % in 2 hours after irradiation whereas on vitronectin in both cells was most promoted by about 160 % in 24 hours after irradiation (Fig. 2C, D).

3. Effects of irradiation on migration capabilities

The results of the migration of both cells in 2, 6, 24 hours after irradiation are shown in Figure 3A. After irradiation, both cells were significantly increased in 24 hours compared with non-irradiated each cell line. On the other hand, in comparison with non-irradiated each cell line, cell migration only in A549R was remarkably promoted in 2 hours after irradiation.

4. Effects of irradiation on cell invasion

The results of the invasion of both cells in 2, 6, 24 hours after irradiation are shown in Figure 3B. Compared with non-irradiated each cell line, after irradiation, invasiveness in both cells was significantly suppressed. However, when compare the temporal variation of both cells, there was no remarkable difference in the feature of the temporal variation of both cells.

5. Effects of irradiation on expression of integrin β1

Figure 4 has shown expression of integrinβ1 in both cells in 2 and 24 hours after irradiation. Integrinβ1 expression was dramatically increased in 24 hours after irradiation. On the other hand, in comparison with non-irradiated each cell line, both cells irradiated in 2 hours were slightly promoted.

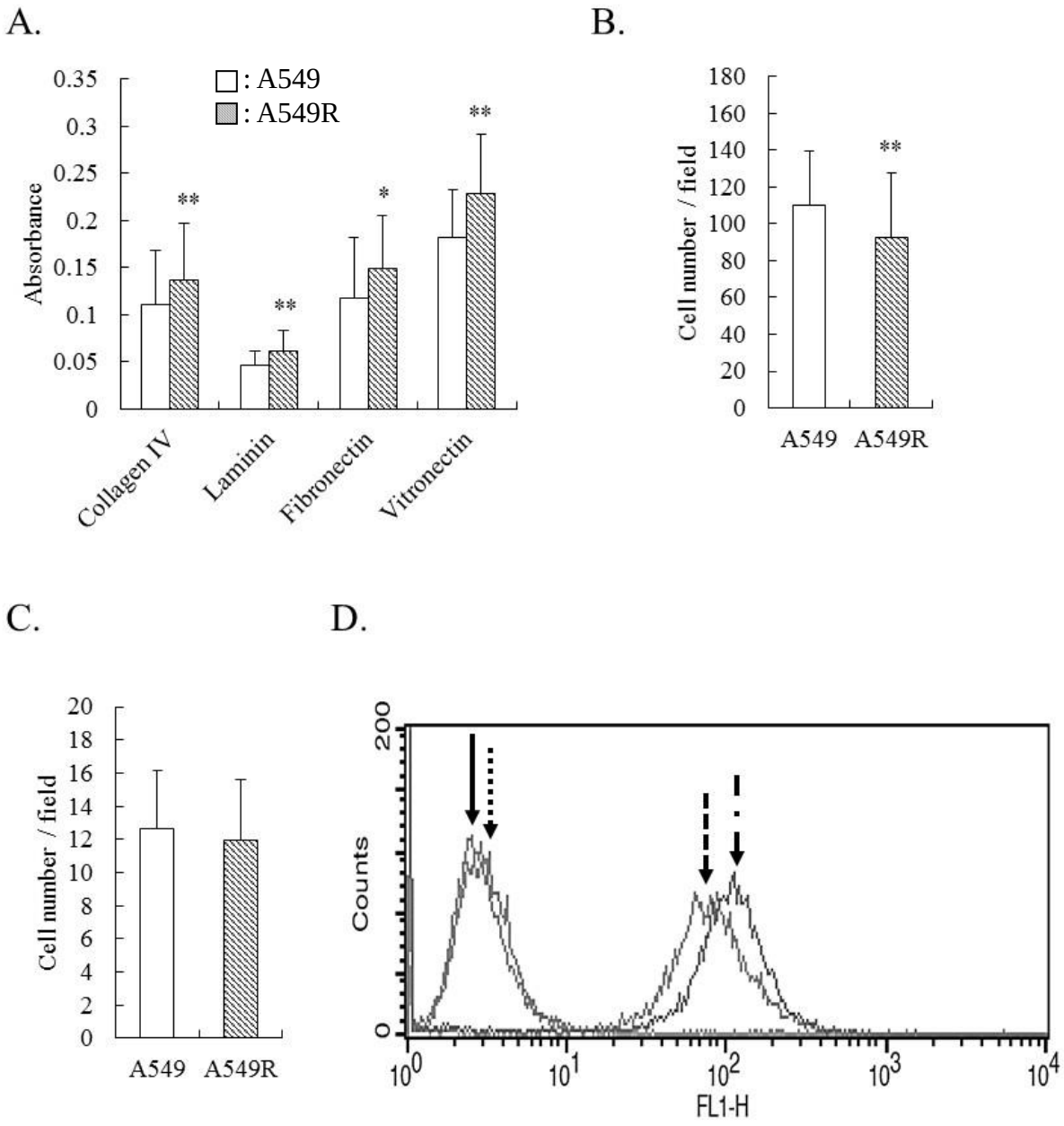

Figure 1. Metastatic potential in both cells, A549 and A549R is examined by cell adhesion assay (A), cell migration assay (B), matrigel invasion assay (C) and integrin β1 expression analysis using flow cytometry (D). Each arrows show below. ⟶ : Negative control in A549. ⇢ : Negative control in A549R. ⇢ : The expression of integrin β1 in A549. ⇢ : The expression of integrin β1 in A549R. **, $p<0.01$, *, $p < 0.05$ (Student's t test, compared with A549).

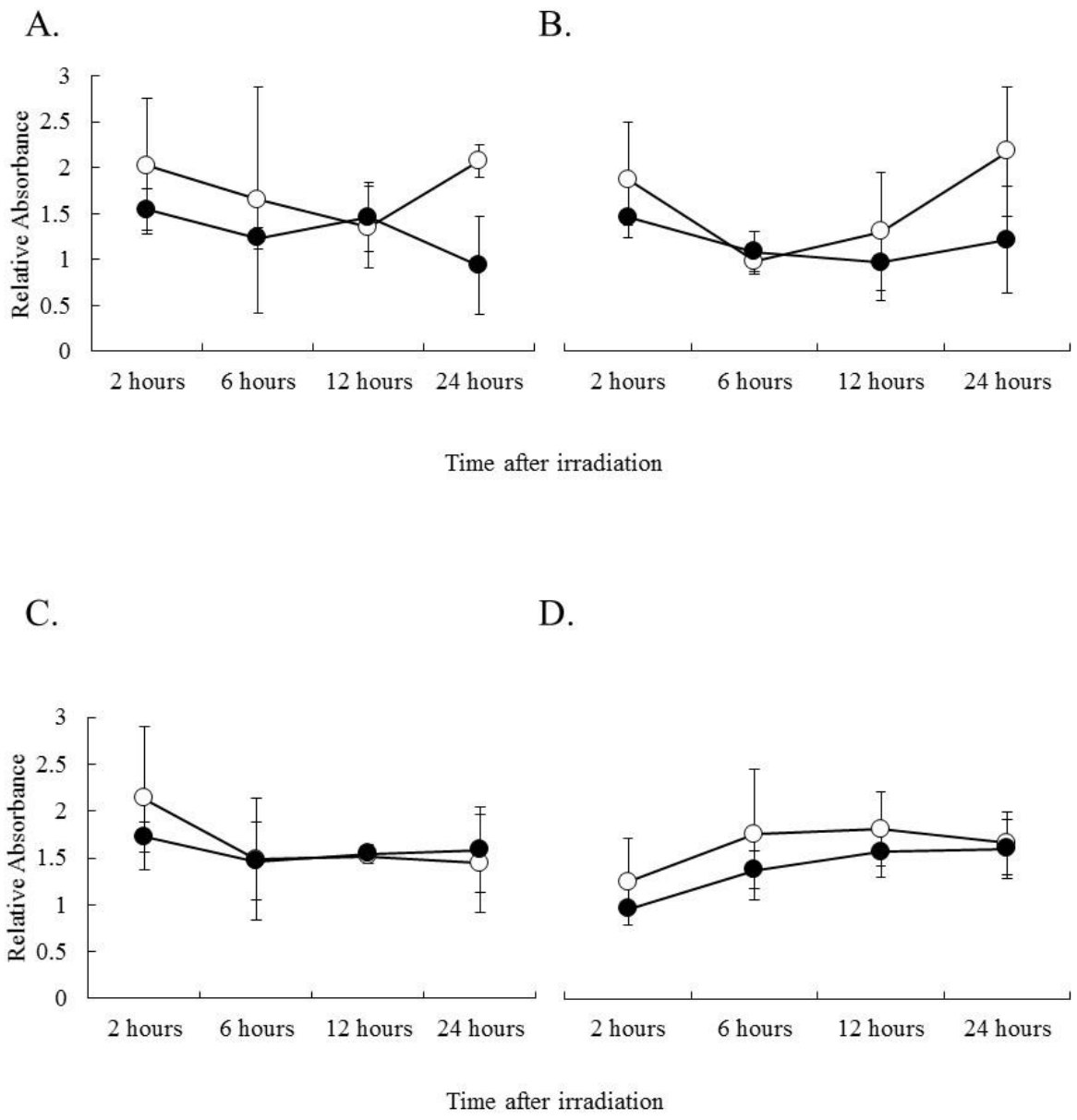

Figure 2. Temporal changes of cell adhesion capability to (A) collagen IV, (B) fibronectin, (C) laminin and (D) vitronectin in A549 or A549R cells after 10 Gy irradiation. The absorbance in each cell after irradiation was averaged and normalized to the absorbance of non-treated each cell. –●– : A549R after irradiation. –○– : A549 after irradiation.

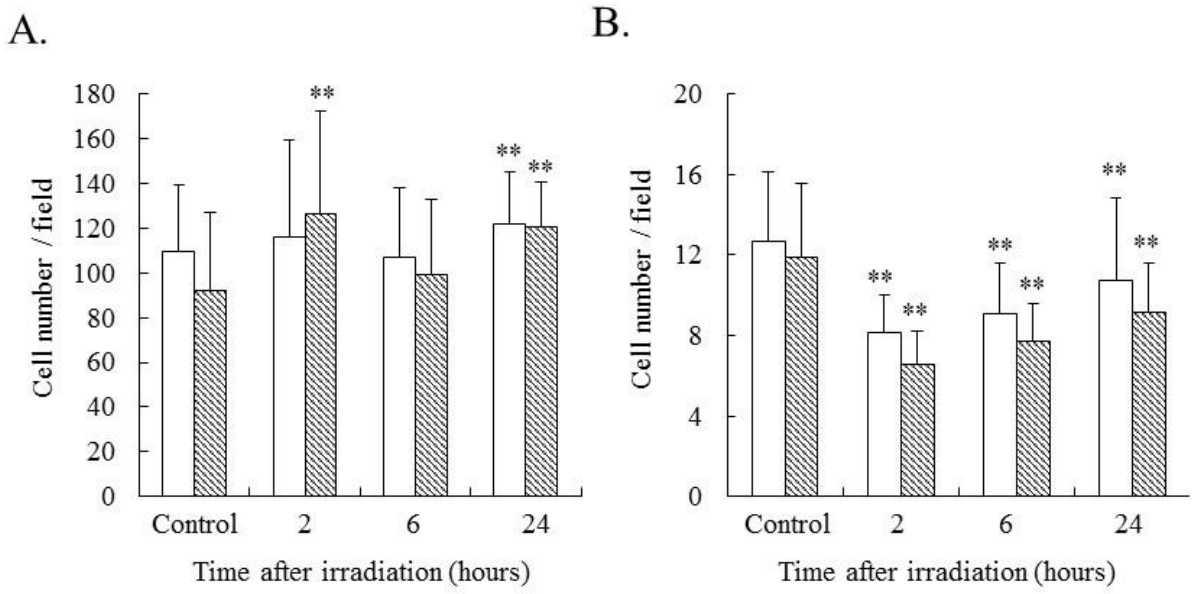

Figure 3. The evaluation of cell migration (A) and cell invasion (B) capability in A549 (□) or A549R (▨) cells after 10 Gy irradiation. **, $p < 0.01$ (Student's t test, compared with untreated cell line).

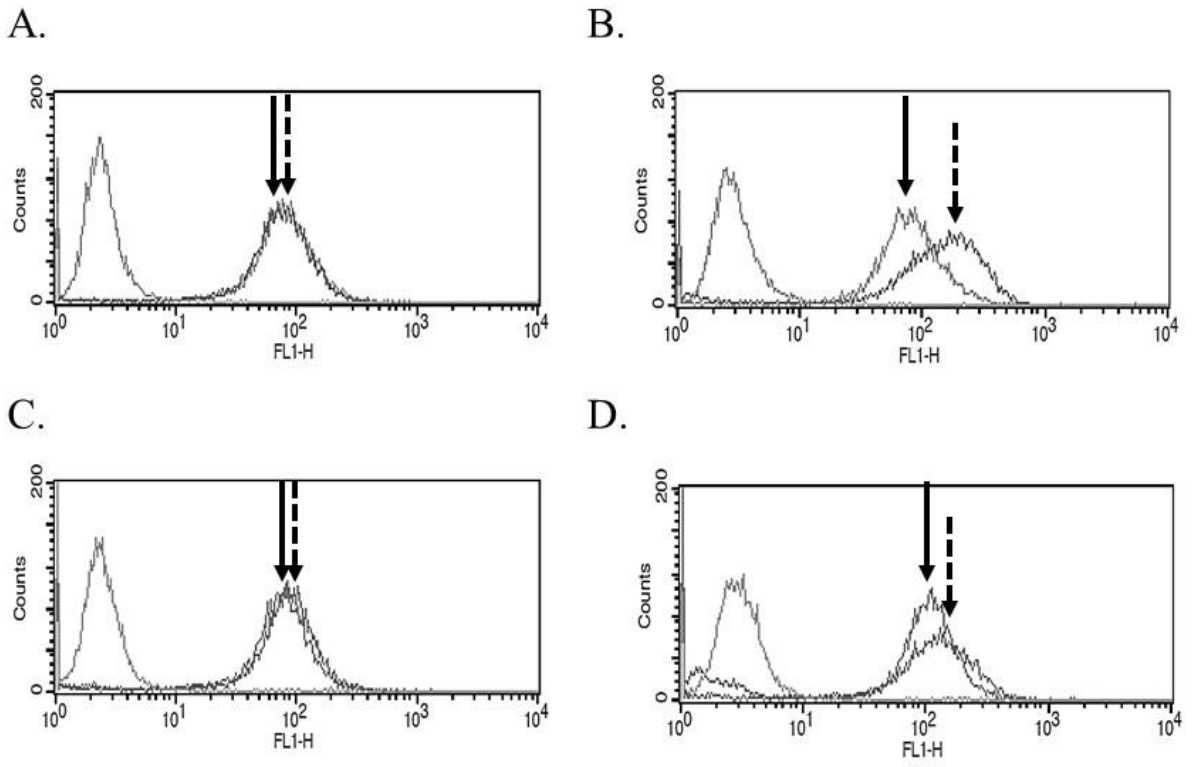

Figure 4. Expression of integrin β1 in A549 cells at 2 hours (A) and 12 hours (B) after 10 Gy irradiation. (C) and (D) show the expression of integrin β1 in A549R cells at 2 hours and 12 hours after 10 Gy irradiation, respectively. Each arrows show below.

→ : Non-irradiation. ⇢ : 10 Gy irradiation.

Discussion

The aim of this study is to examine the metastatic potential of the radio-resistant cancer cell line. First in our study, we performed all experiment under non-irradiated condition to evaluate differences in metastatic potential between both cells. In order to reach distant organs away from primary tumor site, tumor cells have to interact with extracellular matrix, and then enter the lymphatic capillary system or blood flow [6]. Various factors are related to metastatic potentials. Changes in integrin expression level are likely to affect cell functions. Integrin plays an important role to regulate not only the adhesion to extracellular matrix but also cell motility and invasion by controlling cell adhesion [7]. In comparison with A549, expression of integrinβ1 in A549R was increased. This result might cause increase of adhesiveness in A549R. However, cell invasiveness in A549R was not promoted compared with A549. Gliemroth, et al reported that compared with non-irradiated cell line, human glioma cell line which irradiated with 2 Gy/fraction, total 60 Gy dramatically suppressed cell migration and did not change the invasiveness [8]. These results were similar to ours.

Guo, et al has reported that radiation-induced increase of adhesion capacity could be modulated by radiation-induced increase in integrin β1 expression [9]. In addition, Jung et al. reported that irradiation promoted tumor cell motility and focal adhesion formation, and these events already occur in 30 minutes after irradiation and are mediated by increased phosphorylation of FAK [10]. Our findings showed that adhesiveness in both cell lines were most increased in 2 hours or 24 hours compared with non-irradiated each cell line. Radiation-induced increase of adhesive capability in both cells in 24 hours after irradiation was in consistency with radiation-induced increase in integrin β1. However, expression of integrin β1 in both cell lines did not change in 2 hours compared with each non-irradiated cell whereas adhesive capability in both cell lines was significantly promoted after in 2 hours. Tsutsumi et al. reported that the reason why increased adhesion capability after irradiation even though there was hardly difference in expression of integrin β1 in cancer cell after irradiation compared with non-irradiated cell was because ionizing radiation induces the localization of focal adhesion molecules, including integrin β1 [11]. Our results that cell adhesion in both cells in 2 hours after irradiation was increased while expression levels of integrin β1 did not change in both cells after irradiation might denote the same tendency.

Previous findings have suggested that ionizing radiation promotes tumor migration, metastatic and the invasiveness potential of cancer cells [10, 12-14]. The results of present study showed that irradiation remarkably promotes migration in both cells in 24 hours, and cell migration in A549R alone significantly was increased in 2 hours by irradiation. Jung et al. showed that radiation-induced cell migration is due to be activated the p38 MAP kinase pathway and JNK pathway in A549 cells in 30 minutes after irradiation [10]. These pathways are shown to be a prerequisite for promotion of cell migration [15-17]. Our study might show the same tendency.

It is well established that the MMP family plays a key role in metastatic processes, especially

MMP-2 [18-19]. Some studies showed that increased cell invasion after irradiation is due to the enhancement of MMP-2 activity by X-ray irradiation [11-13, 20]. However, we previously reported that cell invasiveness in A549 after 10 Gy irradiation significantly was suppressed [21]. More investigations are needed to elucidate the relevant molecular mechanism behind the different response to ionizing radiation.

Importance of cost-saving aspects

It is a critical problem that medical expenses increase more and more on cancer patients and their families. The share of cancer care in the general practice costs is around 11%. Recently, growth rate of cancer care cost is going up to 42%, more than three times that of national medical expenditure. Performing the radiation therapy efficiently is likely to suppress the cancer care cost growth, and improve the labor productivity: QOL of patients is improved and their rehabilitation goes smoothly.

In summary, our study demonstrates that radio-resistant cancer cell line increase the adhesion capability consistent with enhance the expression integrin β1 and suppress the cell migration. The change in radio-resistant cancer cell line takes place at the time adhesive capacity and cell migration was most increased compared with parental cancer cell line after irradiation. When radio-resistant mechanism becomes clear, a new treatment policy is established. This is likely to shorten the treatment period, thereby reduce medical expenses. In future, it will be necessary to investigate the effective radiotherapy that considers cost-saving aspects of the treatments.

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